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**13. ABSTRACT** ***(Maximum 200 Words)***

The future progress of cancer gene therapy relies on the development of efficient and safe vectors that can deliver therapeutic genes specifically to tumor cells. Using a replication-competent viral vector targeted to tumor cells may be the most efficient way of killing a large number of malignant cells. We intend to develop Sindbis virus (SV), an alphavirus, into a targeted replication-competent viral vector for breast cancer therapy. Since SV kills cells by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to breast cancer cells. To target SV to breast cancer cells, the putative receptor-binding domain(s) of the SV E2 glycoprotein was replaced with the ligand, heregulin, or with an NGR-containing peptide motif that binds to the CD13 receptor expressed on tumor-associated endothelial cells. A heregulin-containing SV preferentially kills a breast cancer cell line that expresses the appropriate receptors. The replication and spread of the heregulin-containing SV vectors is significantly attenuated. However, SV antigen expression and heregulin-E2 glycoprotein expression was detected in BHK cells transfected with SV RNA containing heregulin sequence. We have isolated an NGR-containing SV clone, TE-NGR, that is replication competent in BHK and SLK cells. We are investigating the ability of TE-NGR to preferentially kill cancer cells.

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## Table of Contents

## I. Introduction.

Two main challenges of cancer gene therapy are the development of vectors targeted specifically to tumor cells and the efficient delivery of the therapeutic agent to all or to the majority of tumor cells. Addressing these two issues, we intend to develop Sindbis virus (SV), an alphavirus, into a novel vector for breast cancer gene therapy. The advantages of SV vectors include lack of serious disease caused by SV in humans, the ability of SV to infect nondividing and dividing cells, no risk of insertional mutagenesis because SV is an RNA virus, and the ability to produce high titer stocks and achieve high level of heterologous gene expression. Since SV kills cells by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to tumor cells. Furthermore, use of a replication-competent viral vector will provide a very efficient means of obtaining access to most or all of the tumor cell population. As a first step, erbB-2, a tumor-associated growth factor receptor over-expressed in 20-30 % of human breast carcinomas will be used as a model target. As an additional approach, an NGR-containing peptide motif that binds to the CD13 receptor on tumor vasculature was used to modify the receptor-binding domain of the E2 glycoprotein. The long-term goal of this proposal is to develop target-specific SV vectors for application to breast cancer therapy by modifying the SV E2 envelope glycoprotein with ligands that recognize specific cell surface receptors on breast cancer cells.

## II. Body.

**Technical Objective #1:** Construct viral vectors targeted to breast cancer cells.

**Task 4.** Construct heregulin-containing Sindbis virus (SV) vectors that express the green fluorescent protein (GFP). Construct GFP-expressing SV vectors targeted to tumor vasculature by replacing the putative receptor-binding domains of the E2 glycoprotein with an NGR-containing peptide motif.

We have made numerous plasmid constructs (at least 20) that encode the full-length Sindbis virus genome with sequences for the heregulin epidermal growth factor domain or for the NGR-containing peptide replacing different segments of the putative SV receptor-binding domain. Some of these constructs also express the GFP.

**Technical Objective #2:** Demonstrate the ability of the targeted SV vectors to infect and kill human breast cancer cells.

**Task 5:** Test the SV vectors for the ability to infect and spread in various breast cancer cell lines by transfecting viral RNA and observing GFP expression using fluorescence microscopy. Determine the degree of cell death caused by the targeted SV vectors using FACS analysis with propidium iodide staining.

1) **Detection of GFP expression in transfected cancer cell lines.** We transfected a baby hamster kidney cell line (BHK-21), a breast cancer cell line (SKBR3), and a Kaposi's sarcoma cell line (the SLK cell line--this cell line expresses the appropriate receptor for the NGR-containing peptide) with heregulin-containing and NGR-containing SV-vector RNAs that express GFP. Our goal was

to observe the ability of the viral vectors to spread in and replicate in a cell line that is very permissive to SV infection (the BHK cell line) and in the SKBR3 and SLK cancer cell lines. We observed the transfected cancer cell lines using fluorescence microscopy and also performed flow cytometry to detect GFP-expressing cells. The viral vectors were significantly attenuated in all of the cell lines indicating that substitution of the receptor-binding domain with the ligands impaired viral replication. In BHK cells transfected with the NGR-containing viral vector RNA, only single BHK cells expressed the GFP without evidence of spread over time (appendix B; figures 1 to 3). Transfection of BHK cells with the control viral vector RNA, containing the wild-type structural genes, resulted in the majority of BHK cells expressing the GFP and the eventual death of the monolayer (figure 2). Transfection of BHK cells with the NGR-containing SV vector RNAs did not result in a significant amount of death of the BHK cell monolayers.

We could not detect GFP expression in SLK cells or SKBR3 cells transduced with our wild-type control SV vector RNA despite the induction of death in these cultures by this vector. We also could not detect GFP expression after transfection of these cells with the "targeted" viral vector RNA. We could not distinguish the signal emitted by the GFP from the background autofluorescence produced by the tumor cells, the medium and/or the culture vessels. We used various techniques to try to diminish the autofluorescence but were still unable to detect GFP expression.

BHK cells were transfected with heregulin-containing SV RNA. Indirect immunofluorescence microscopy was performed using anti-SV rabbit serum and anti-heregulin serum (Appendix B; figures 4 and 5). These assays demonstrated the expression of SV antigens and of the heregulin-E2 protein.

Our original hypothesis stated that a replication-competent SV vector, a vector with the ability to spread through the tumor, would be developed in order to try to infect the majority of cancer cells in a tumor. As described above, the heregulin-containing and NGR-containing SV vectors are quite attenuated without evidence of significant spread of the vectors in vitro. This hurdle potentially can be overcome by serial passaging of the vectors in the appropriate target cell lines in order to try to select for a virus that is not quite as attenuated and that is able to replicate in breast cancer cells. We have evidence that this approach can work because we have isolated an NGR-containing SV clone (TE-NGR) that is able to replicate in BHK cells and in the SLK Kaposi's sarcoma cell line as efficiently or more efficiently than the parent virus from which it was derived (appendix B; figure 6). This clone most likely has a naturally occurring mutation that enables it to replicate in BHK cells and in certain cancer cell lines. We plan to sequence the E1 and E2 glycoproteins of this clone to try to identify mutations that enable replication in certain cancer cell lines.

2) **Assessment of the permissiveness of the cancer cell lines to wild-type SV infection.** The inability to detect viral vector replication and GFP expression in the SKBR3 and SLK cancer cell lines prompted us to determine the permissiveness of these particular cell lines to wild-type SV infection. SKBR3 cells were infected with the wild-type SV, TE, at a multiplicity of infection (MOI) of 10 (note: the experiments described in paragraph number 1 were performed using viral RNA because virus stocks of the "targeted" virus with significant viral titers could not be produced; this issue was discussed in the previous annual report). Forty-eight hours after infection, the SKBR3 cells were stained with anti-SV rabbit serum and with a FITC-conjugated anti-rabbit antibody. The amount of cells expressing SV antigens (E1 and E2 glycoproteins) on their surface was assayed using a flow cytometer. Twenty-two percent of TE-infected SKBR3 cells expressed

SV antigens on their surface (figures 7 and 8). Indirect immunofluorescence microscopy of TE-infected SKBR3 cells also revealed production of viral antigens (figure 9). These data demonstrate that the SKBR3 human breast cancer cell line is permissive to infection by SV.

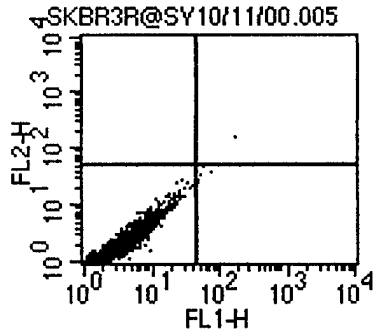


**Figure 7**. SKBR3 cells infected with TE virus at an MOI = 10 and stained with normal rabbit serum.

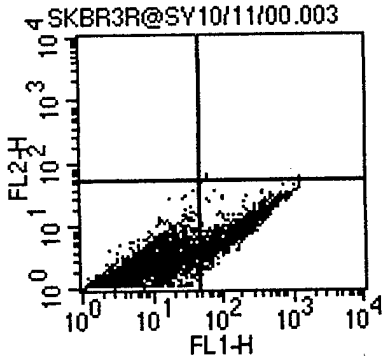


**Figure 8**. SKBR3 cells infected with TE virus and stained with anit-SV rabbit serum.

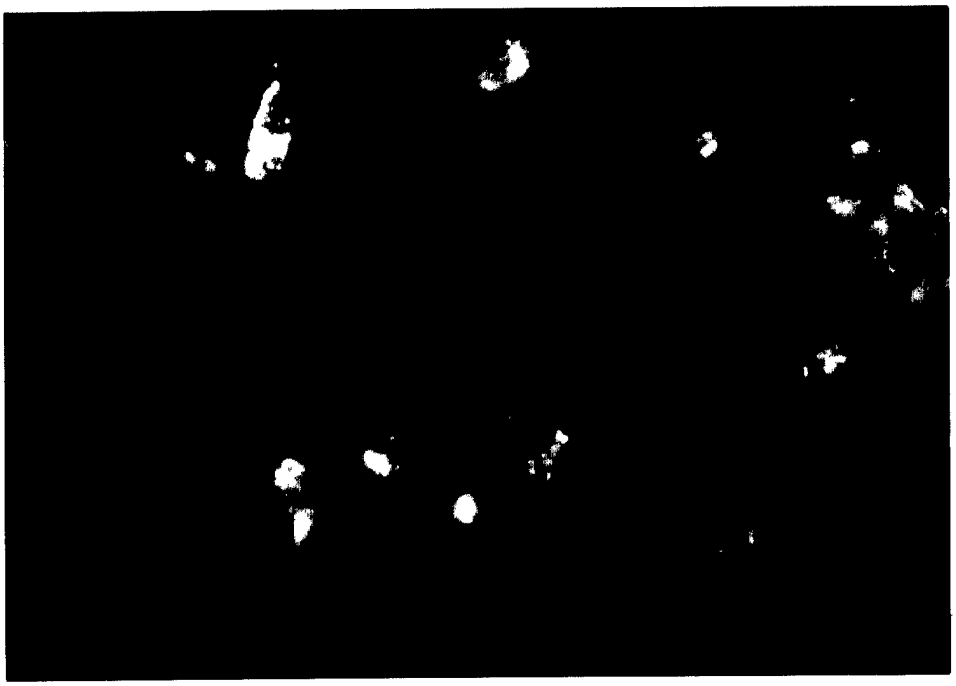

**Figure 9**. Indirect immunofluorescence microscopy of TE-infected SKBR3 cells. Anti-SV rabbit serum.

SLK cells were infected with TE at an MOI = 1. A propidium iodide assay was performed to determine the number of cells killed by TE virus infection. At 6 days after infection, 22% of TE-infected SLK cells were dead compared to 10% of mock-infected cells (figures 10 and 11). Indirect immunofluorescence microscopy of TE-infected SLK cells revealed that these cells readily express SV antigens (figure 12). In toto, these results demonstrate that the SKBR3 and SLK cell lines are permissive to SV infection and that the inability to detect spread of the heregulin-containing and NGR-containing SV vectors was not a result of the non-permissiveness of SKBR3 and SLK cell lines to SV infection. The inability to detect GFP expression and lack of spread of the vectors may be a result of

the low transfection efficiency in these cancer cell lines (5%) combined with the attenuated replication of the vectors.

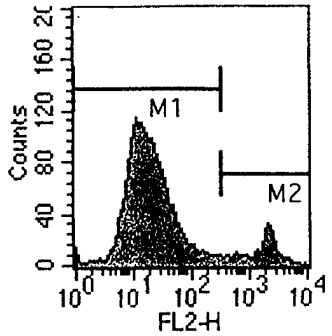


**Figure 10**. Propidium iodide assay of mock-infected SLK cells. M2 = dead cells

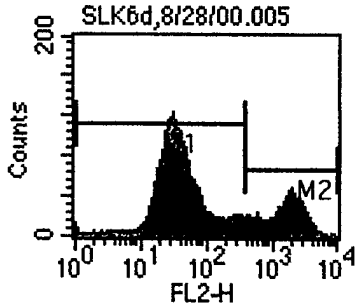


**Figure 11**. Propidium iodide assay of TE-infected SLK cells. M2 = dead cells.

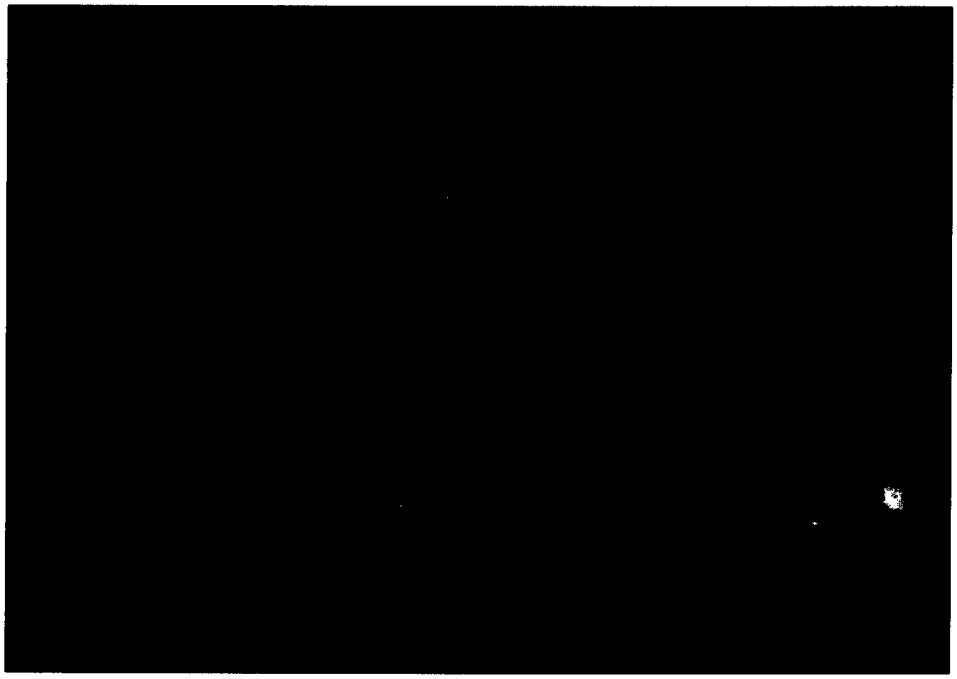

**Figure 12**. Indirect immunofluorescence microscopy of TE-infected SLK cells using anti-SV rabbit serum.

3) **Growth of SV in cancer cell lines.** To further characterize the replication of the wild-type or parent (the virus from which the above clones are derived) viruses in SKBR3 cells, we generated one-step growth curves. SKBR3 cells were infected with TE or the parent virus (lkd102.10) at an MOI = 5 and 200 µl aliquots of supernatant fluids were removed from the cultures at regular intervals. Plaque assays were performed using the supernatant fluids and the amount of plaque forming units per milliliter was determined. (figure 13). The viruses reached titers of $10^6$ in this cell line, confirming the permissiveness of the SKBR3 cell line to SV infection. We are in the process of generating growth curves in the SLK cell line.

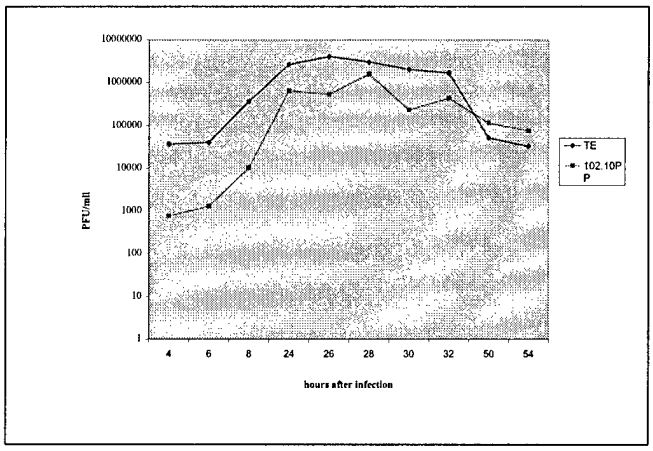


**Figure 13.** Viral growth curve of TE (wild-type)- and lkd102.10 (parent)-infected SKBR3 cells.

4) **Preferential killing of SKBR3 cells with a heregulin-containing SV vector.** We have previously demonstrated that the heregulin-containing SV vectors preferentially kill SKBR3 cells when compared to BHK cells (please see annual report submitted in October 1999; Atlanta abstract).

5) We performed competition experiments with a GST-heregulin fusion protein (produced in our laboratory) and with an anti-HER3 heregulin-blocking antibody (Labvision, CA) to determine whether the preferential killing of SKBR3 cells was occurring as a result of a specific interaction between the heregulin-ligand expressed by the virus and the HER receptors on SKBR3 cells. Neither the presence in SKBR3 cultures transfected with the heregulin-containing SV RNA of the GST-heregulin fusion protein nor the anti-HER-3 heregulin-blocking antibody was able to alter the death induced by the heregulin-containing viral RNA (figures 14 and 15). These data indicate that perhaps the preferential killing of SKBR3 cells by the heregulin-containing viral RNA is not occurring as a result of an interaction between the heregulin ligand and the appropriate HER receptors but through a different mechanism. Perhaps the heregulin-containing E2 glycoprotein triggers a death pathway cascade after the heterologous protein is translated in the cytoplasm.

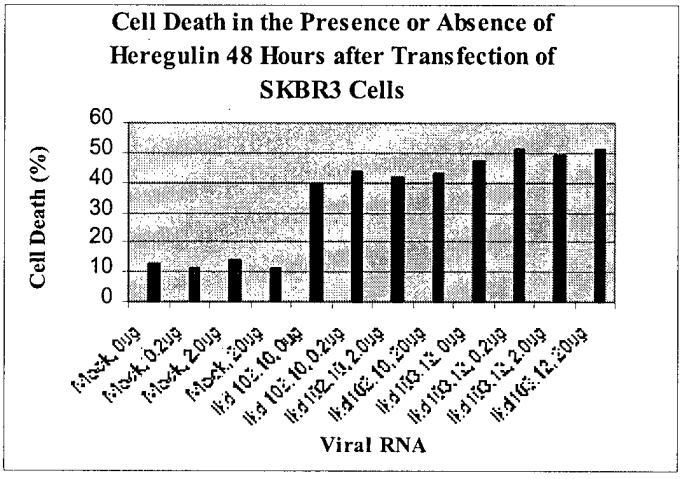


**Figure 14.** lkd102.10 is the parental virus RNA and lkd103.12 is the heregulin-containing SV RNA. 0, 0.2, 2, or 20 μg of heregulin were added to the cultures.

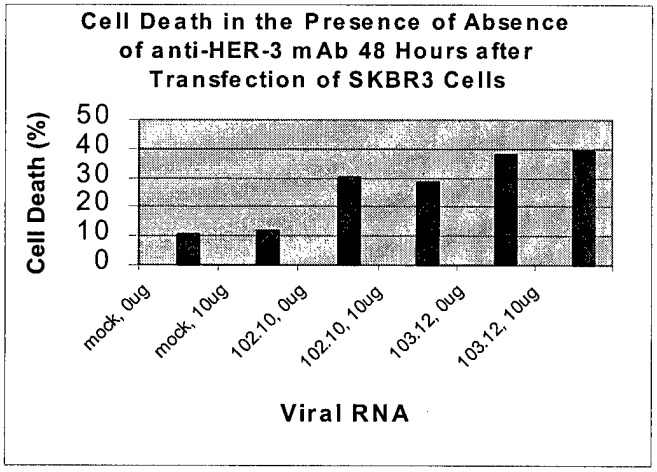


**Figure 15.** lkd102.10 is the parental virus RNA and lkd103.12 is the heregulin-containing SV RNA. 0 or 10 μg of anti-HER-3 heregulin-blocking antibody was added to the cultures.

**Technical objective #3:** Test the targeted viral vectors in a nude mouse model of breast cancer.

We have just begun our animal experiments, so we do not have any data to report at this time.

## III. Key Research Accomplishments.

- Constructed SV plasmids or SV vectors expressing EGFP that have heregulin or the NGR-containing peptide cloned into different locations of the putative receptor-binding domain of the E2 glycoprotein.
- Demonstrated that the wild-type and parent SVs can replicate in the cancer cell lines we are studying. However, the "targeted" viruses are significantly attenuated in their replication in these cell lines except for one NGR-containing clone, TE-NGR.
- Performed experiments to determine whether the preferential killing of SKBR3 cells by the heregulin-containing clones is occurring as a result of a specific interaction between heregulin and the HER receptors expressed on the surface of the SKBR3 cells.

## IV. Reportable Outcomes.

**Abstract.** Preferential Killing of Breast Cancer Cells by a Heregulin-containing Sindbis Virus Vector. Lesia K. Dropulic, J. Marie Hardwick, Jennifer L. Nargi. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta, Georgia, June 8-11, 2000.

**Manuscript in preparation.** Lesia K. Dropulic, J. Marie Hardwick, Holly L. Thomas. Preferential Killing of Breast Cancer Cells with an Heregulin-containing Sindbis virus.

**U.S. Patent Application.** Use of Targeted Sindbis Virus Vectors to Treat Cancer, submitted July, 1998. Rebutted 7/00.

## V. Conclusions.

The experiments performed during the course of this project have served as a starting point to determine whether it is feasible to develop a replication-competent viral vector that is targeted to breast cancer cells. We have learned that using larger ligands, such as the epidermal growth factor-like domain of heregulin (200bp), to replace the receptor-binding domain of SV can result in significant impairment of viral replication. We have not replaced every potential receptor-binding site of E2, so there are still other sites to replace that may be less critical for viral replication. Using smaller ligands, such as the 13 amino acid NGR-containing peptide, may prove to be more successful, as evidenced by the fact that we have isolated a replication-competent NGR-containing SV clone. We are in the process of analyzing this clone in terms of its ability to preferentially infect and kill the SLK Kaposi's cell line that bears the appropriate receptor. Testing these viral vectors in vivo in nude mouse models is critical to determine the effect on breast cancer tumor formation and growth.

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**Kasahara N, Dozy AM, Kan YW.** Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. Science 1994; 266: 1373-1376.

**Mebatsion T, Finke S, Weiland F, Conzelmann K-K.** A CXCR4/CD4 pseudotype rhabdovirus that selectively infects HIV-1 envelope protein-expressing cells. Cell 1997; 90: 841-847.

**Appendix A.**

**Personnel receiving pay from the research effort:** Lesia K. Dropulic, M.D.

## VII. Appendix B.

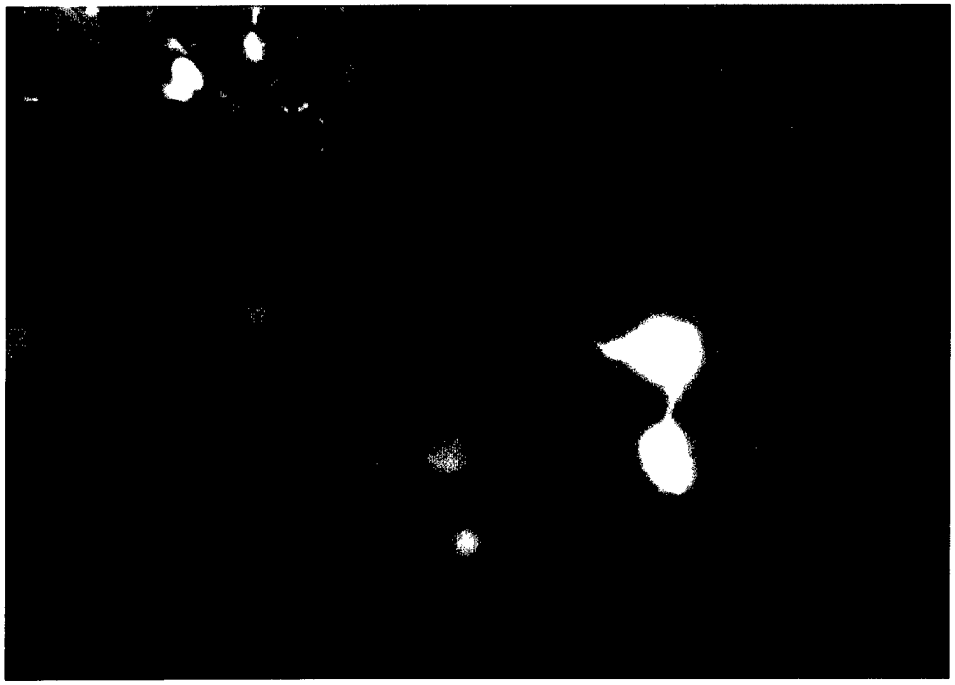

**Figure 1.** BHK cells transfected with NGR-containing SV RNA-EGFP.

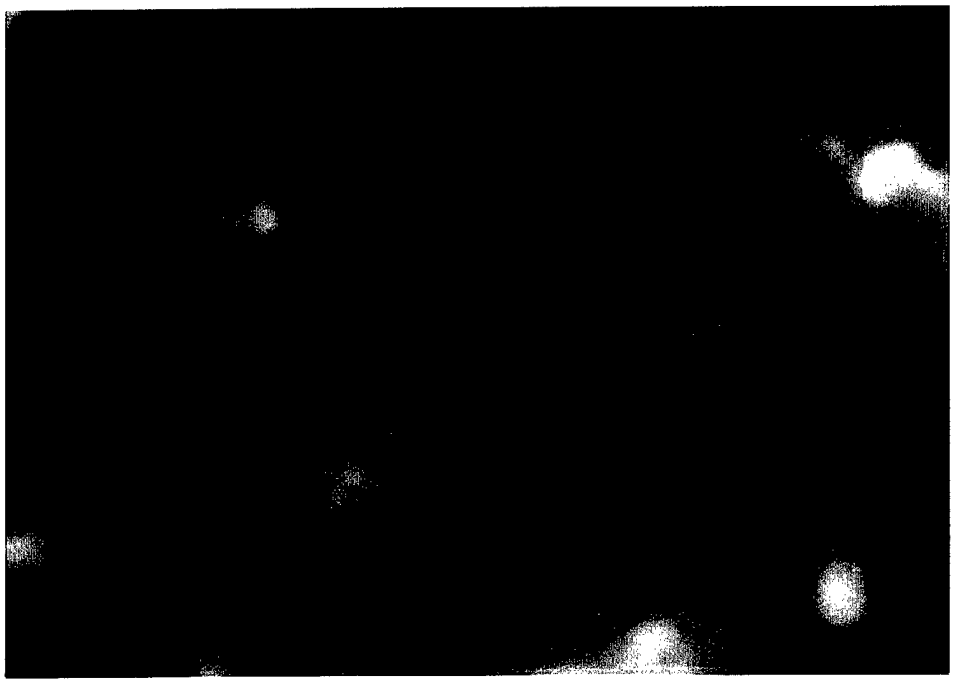

**Figure 2.** BHK cells transfected with wild-type SV vector RNA, dsTE-EGFP.

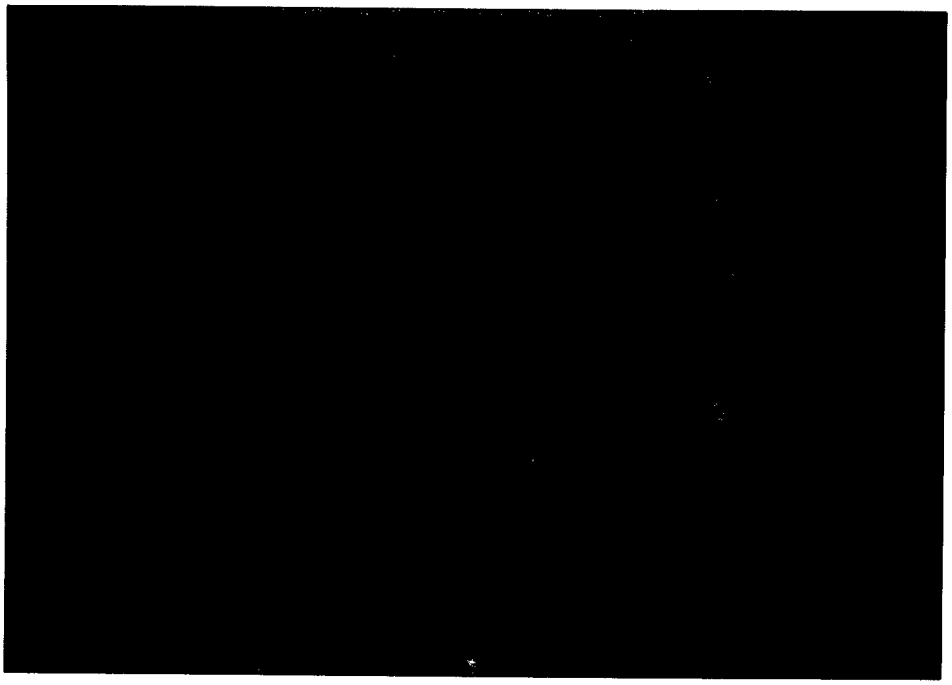

**Figure 3.** Mock-transfected BHK cells.

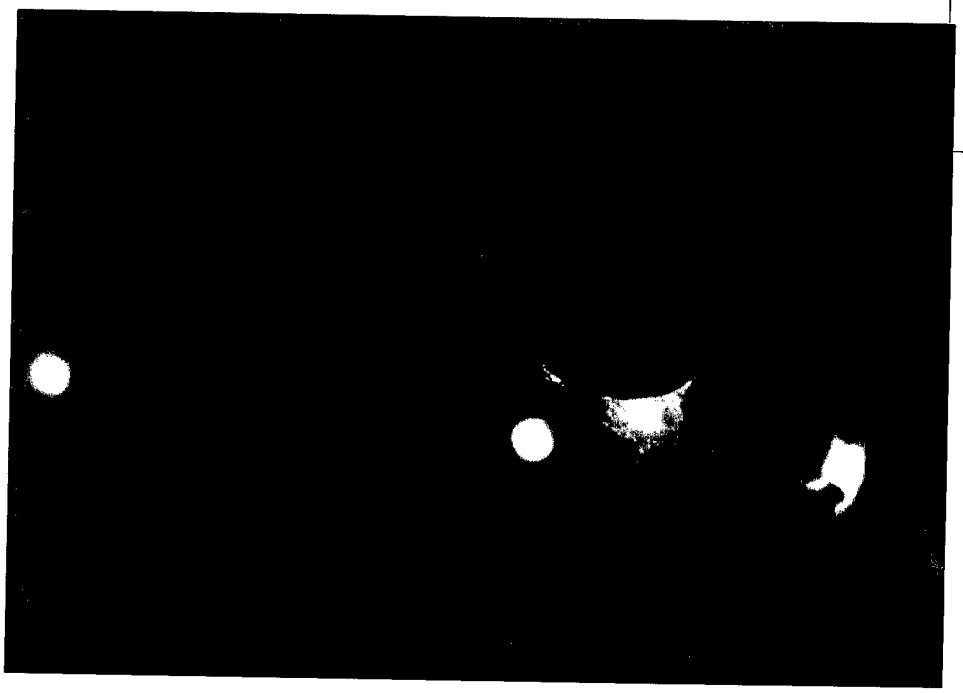

**Figure 4.** BHK cells transfected with heregulin-containing SV RNA. Indirect immunofluorescence microscopy with anti-SV rabbit serum.

**Figure 5.** BHK cells transfected with heregulin-containing SV RNA. Indirect immunofluorescence microscopy with anti-heregulin antibody.

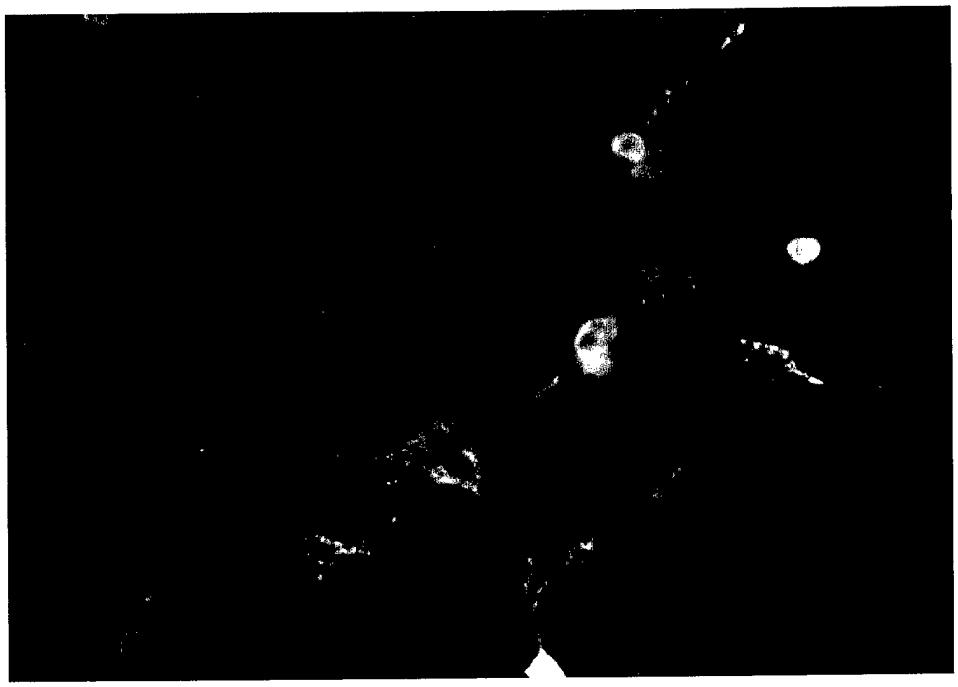

**Figure 6.** BHK cells infected with TE-NGR. Similar results were seen in SLK cells.

# PREFERENTIAL KILLING OF BREAST CANCER CELLS BY A HEREGULIN-CONTAINING SINDBIS VIRUS VECTOR

**Lesia K. Dropulic[1], J. Marie Hardwick[1, 2], Jennifer L. Nargi[2]**

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The future progress of cancer gene therapy relies on the development of efficient and safe vectors that can deliver therapeutic genes specifically to tumor cells. Using a replication-competent viral vector targeted to tumor cells may be the most efficient way of specifically killing a large number of malignant cells. We intend to develop Sindbis virus (SV), an alphavirus with a positive sense RNA genome, into a targeted replication-competent viral vector for breast cancer gene therapy. Since SV kills cells by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to breast cancer cells.

We are attempting to target Sindbis virus to breast cancer cells by replacing the putative receptor-binding domain of the E2 glycoprotein with the ligand, heregulin. Heregulin has affinity for breast cancer cells that express the human epidermal growth factor receptors, HER-2, HER-3, and HER-4. SKBR3 breast cancer cells and baby hamster kidney cells, BHK-21, were transfected with genomic SV RNAs containing the heregulin sequence in place of a portion of the putative receptor-binding domain of the E2. The percentage of dead cells in the cultures was determined by the trypan blue exclusion assay 48 hours after transfection. The parental SV was able to replicate in BHK (80% dead cells) and SKBR3 cells (20% dead cells), albeit less efficiently in SKBR3 cells. Transfection of the heregulin-containing viral RNAs caused significant cell death only in SKBR3 cells (25%) and not in BHK cells. Cytopathic effect was evident only in SKBR3 cells.

Preferential killing of the MCF-7 breast cancer cell line was observed with a heregulin-containing SV that was complemented with a defective SV helper RNA. MCF-7 breast cancer cells were infected with parent SV or with a complemented heregulin-containing virus. At 74 hours after infection, a propidium iodide assay using a flow cytometer was performed to determine the percentage of MCF-7 cells that was killed. The heregulin-containing SV killed more MCF-7 cells (42%) compared to parent virus (20%) and to mock-infected cultures (6% dead cells).

These data demonstrate that a recombinant SV can preferentially kill breast cancer cell lines expressing the human epidermal growth factor receptors. These data support the hypothesis that SV can be reengineered into a tumor-specific, replication-competent virus by replacing the receptor-binding domain with a ligand that recognizes a specific receptor on the surface of a tumor cell.

The U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7073 supported this work.